HYDROGEN SULFIDE THERAPY IMPROVES INTESTINAL RECOVERY THROUGH ENDOTHELIAL NITRIC OXIDE DEPENDENT MECHANISMS

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Amanda Jensen

HYDROGEN SULFIDE THERAPY IMPROVES INTESTINAL RECOVERY THROUGH ENDOTHELIAL NITRIC OXIDE DEPENDENT MECHANSISMS

H_2S is a gaseous mediator that acts as an anti-inflammatory agent contributing to gastrointestinal mucosal defense. It promotes vascular dilation, mucosal repair, and resolution of inflammation following intestinal ischemia and may be exploited as a novel therapeutic agent. It is unclear if H_2S works through nitric oxide-dependent pathways in the intestine. We appreciated that H_2S was able to improve post-ischemic recovery of mesenteric perfusion, mucosal integrity, and inflammation. The beneficial effects of H_2S appear to be mediated through endothelial nitric oxide-dependent pathways.

Troy A. Markel, MD, Chair

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LIST OF ABBREVIATIONS

AMI……acute mesenteric ischemia

eNOS……endothelial nitric oxide synthase

eNOS KO……..endothelial nitric oxide synthase knock out

FGF-2 ……...fibroblast growth factor 2

G-CSF ……granulocyte colony stimulating factor

HGF …….hepatic growth factor

H_2S……...hydrogen sulfide

IL-6 ……. interleukin 6

IL-9 ……..interleukin 9

IL-10 …….interleukin 10

IP-10 ……interferon gamma induced protein

I/R…….ischemia-reperfusion

KC …….keratinocyte chemoattractant

LDI…….laser doppler imager

MIP-1α ……macrophage inflammatory protein 1 alpha

MIP-2 …….macrophage inflammatory protein 2

NaHS……Sodium Hydrosulfide

ROI……..region of interest

SMA ……...superior mesenteric artery

VEGF ……vascular endothelial growth factor

WT……wild type

CHAPTER ONE: INTRODUCTION

1.1 Mesenteric Ischemia

Acute mesenteric ischemia (AMI) continues to be a devastating intra-abdominal emergency with mortality as high as 60-80% [1]. It is caused by either 1) a sudden acute arterial occlusion, 2) a venous occlusion or 3) a sudden drop in circulating pressure. During hypoperfusion, this insufficient blood flow within the mesenteric circulation is unable to meet intestinal metabolic demands and often this may lead to mesenteric infarction and intestinal necrosis. Patients who remain untreated can quickly decompensate and progress to shock, multi-system organ failure and death [2]. The most critical factor that continues to impact outcomes in patients is the time to diagnosis and intervention.

Major etiologies of intestinal ischemia include arterial obstruction with embolic occlusion (40-50%), arterial thrombotic occlusion (15-25%), and venous thrombosis (5%) with the remaining due to non-occlusive ischemia that causes intestinal hypoperfusion [3-5]. This disease affects multiple patient populations with varying ages and comorbidities and accounts for about 0.1% of all hospital admissions [5].

If patients survive these ischemic episodes, they are often faced with prolonged hospitalization and long term parenteral nutrition needs [6]. Additionally, reported overall survival at one, three and five years following surgery for AMI has been found to be 26%, 23%, and 21% respectively [7]. Early diagnosis and aggressive therapy may significantly reduce the morbidity and mortality of this life-threatening disease. While clinical studies emphasize diagnostic and therapeutic algorithms to expedite treatment for the diagnosis of AMI, the disease prognosis remains dismal. To that end, noteworthy advancements in the medical treatment of intestinal ischemia within the last decade have been sparse and a novel therapeutic option for the treatment of intestinal ischemia is of the utmost importance.

1.2 Hydrogen Sulfide

Hydrogen sulfide (H_2S) has recently been identified as a potential therapeutic strategy for gastrointestinal disease. H_2S donors have been found to be cytoprotective in the setting of NSAID-induced intestinal injury through modulation of the bile and the microbiome [8, 9]. Additionally, in the setting of colitis, endogenous H_2S production increases and is found to decrease following resolution of colitis, while exogenous application also causes significant reduction in the severity of colitis [10].

H_2S has also been found to be of benefit in intestinal IR injury. Pre-conditioning with an H_2S donor prior to intestinal ischemia has been found to prevent mitochondrial dysfunction by a BK_{Ca} channel-dependent mechanism [11]. In the post-ischemic period, application of an H_2S donor decreases apoptosis and preserves intestinal architecture [12]. Other I/R-related injuries including myocardial infarction, cerebral ischemia, and transplant associated renal ischemia have also observed therapeutic benefit and end-organ protection with use of H_2S donors [13-16]. H_2S likely plays an important role physiologically through cellular signaling and vasodilation. Additionally, it exhibits anti-inflammatory, anti-oxidant and anti-apoptotic effects [11, 12, 17] (Figure 1).

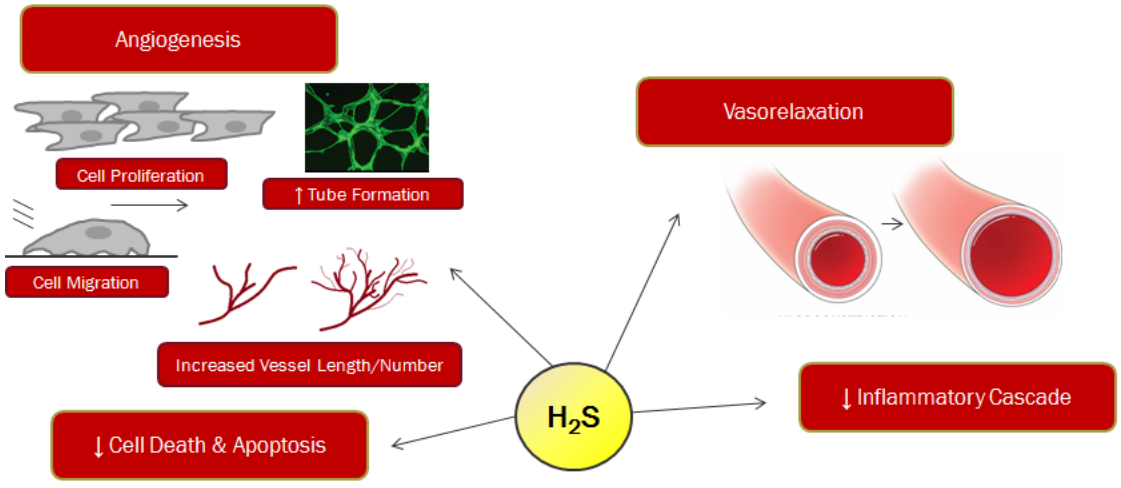

Figure 1. Protective Effects of H_2S

1.3 Mechanisms of Action

Hydrogen sulfide is a gaseous mediator that acts as an anti-inflammatory agent contributing to gastrointestinal mucosal defense. It promotes vascular dilation, mucosal repair, and resolution of inflammation following intestinal ischemia.H_2S may work through endothelial nitric oxide synthase (eNOS) to bring about its protective effects by increasing the production of nitric oxide; a known vasodilator. H_2S can donate a sulfur moiety to a cysteine on eNOS which then promotes its dimerization [18] (Figure 2). Only once dimerized can eNOS facilitate the production of nitric oxide [19]. Nitric oxide, as a downstream effector, may then promote vascular dilation[20].

Other studies on coronary artery physiology have suggested that hydrogen sulfide may exert its effects through both nitric oxide-dependent and nitric oxide-independent pathways, depending on native vascular tonicity [21]. Testai et al. specifically demonstrated that in normotensive rats, hydrogen sulfide worked through nitric oxide-dependent pathways, but in hypertensive rats, hydrogen sulfide mediated its effects through nitric oxide-independent pathways.

Currently, there is no clear understanding of how hydrogen sulfide mediates its protective effects in the intestinal mesenteric vascular bed. Therefore, prior to hydrogen sulfide therapy being used in a clinical setting, the downstream pathways of this novel treatment must be further elucidated.

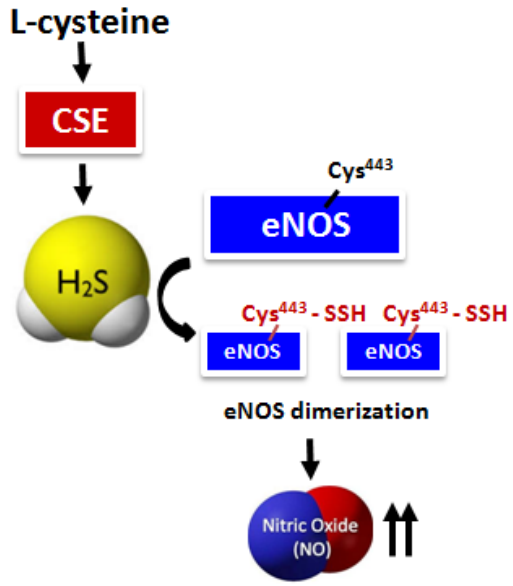

Figure 2. Proposed post-translational H_2S Modification of eNOS. H_2S S-sulfhydration of eNOS at Cys443 to stabilize eNOS dimers. This in turn leads to increased NO production and decreased superoxide generation, both of which are essential to maintain normal endothelial functions. ([18] Adapted from Altaany et al. Sci Signal, 2014. **7**(342): p. ra87)

1.4 Impact as a Treatment for Ischemia

In the United States alone, there are over 47,000 cases of bowel ischemia per year [22]. This accounts for an annual cost upwards of over 600 million [22]. Currently, early diagnosis is critical with mortality increasing significantly following intestinal infarction. To this end, duration of symptoms is predictive of mortality with patients having <12 hours of symptoms found to have a mortality rate of 0-17%, patients with symptoms for <24 hours having a mortality rate of 44-57% and patients with symptoms for >24 hours having mortality rates as high as 73-95%[23].

Current treatment therapies include embolectomy, revascularization, and resection. To date however, there have been no innovative treatment modalities aimed at recovering the infarcted bowel. Therefore, a novel therapeutic treatment is essential to aid in improving outcomes in this patient population. In this regard, the use of hydrogen sulfide (H_2S) has recently been identified as a potential therapeutic strategy for gastrointestinal disease.

1.5 Purposes and Aims of Studies

Although the exact mechanisms of H_2S therapy is not entirely understood, there is a significant amount of literature confirming the therapeutic benefits of H_2S in the setting of I/R injury. Approaching this project, we primarily sought to determine if H_2S would ameliorate intestinal I/R injury. Furthermore, additional questions had stemmed from how little we know about the exact mechanisms of action.

Currently, there is no clear understanding of how hydrogen sulfide mediates its protective effects in the intestinal mesenteric vascular bed. Therefore, prior to hydrogen sulfide therapy being used in a clinical setting, the downstream pathways of this novel treatment must be further elucidated. We hypothesized that: 1) H_2S would improve post-ischemic survival, mesenteric perfusion, intestinal mucosal injury scores, and intestinal inflammation more effectively compared to vehicle following intestinal I/R, and 2) the benefits of H_2S therapy would be mediated through endothelial nitric oxide synthase-dependent pathways.

CHAPTER TWO: MATERIALS AND METHODS

2.1 Murine Intestinal Ischemia-Reperfusion Model

Experimental protocols and animal use were approved by the Indiana University Institutional Animal Care and Use Committee. Wild-type adult male mice (C57BL/6J, Stock No: 00664, 8-12 weeks/20-30g Jackson Laboratory, Bar Harbor, ME) and eNOS KO mice (B6.129P2-Nos3tm1Unc/J, Stock No: 002684, 8-12 weeks/20-30g; Jackson Laboratory, Bar Harbor ME) underwent at least 48 hours of acclimation to the new environment prior to experimentation. Mice were provided normal chow and water and kept in 12 light/12 dark cycle housing.

Mice were anesthetized using 3% isoflurane followed by maintenance at 1.5% isoflurane in oxygen. A heating pad was used to achieve temperature homeostasis and the abdomen was prepped using a hair removal lotion followed by sterile preparation with 70% ethanol and betadine (Figure 3). To account for intra-operative fluid losses, one milliliter of 0.9% normal saline was injected subcutaneously pre-operatively. All animals were given analgesia (1mg/kg buprenorphine and 5mg/kg carprofen) by subcutaneous injection pre-operatively.

Under sterile conditions, a midline laparotomy was performed and the intestines were eviscerated. The base of the superior mesenteric artery was identified and clamped using an atraumatic microvascular clamp. The intestines were then placed back into the abdominal cavity and the abdomen was temporarily closed using silk suture to prevent evaporative losses. Following 60 minutes of intestinal ischemia, the abdomen was reopened and the atraumatic clamp was removed. The abdominal fascia and skin were then closed in a two-layer fashion with silk suture. Prior to complete abdominal closure the animals underwent intraperitoneal injection with 250µL of phosphate buffered saline (vehicle control), or 250µL of sodium hydrosulfide (NaHS) at doses of 2 nmol/kg ($1.12x10^{-4}$mg/kg) or 2µmol/kg ($1.12x10^{-1}$mg/kg) in PBS. Triple

antibiotic ointment was applied to the abdominal incision following complete closure. Following surgery, animals were placed in a cage on a heating pad and allowed to awaken. Once fully recovered, animals were returned to animal housing.

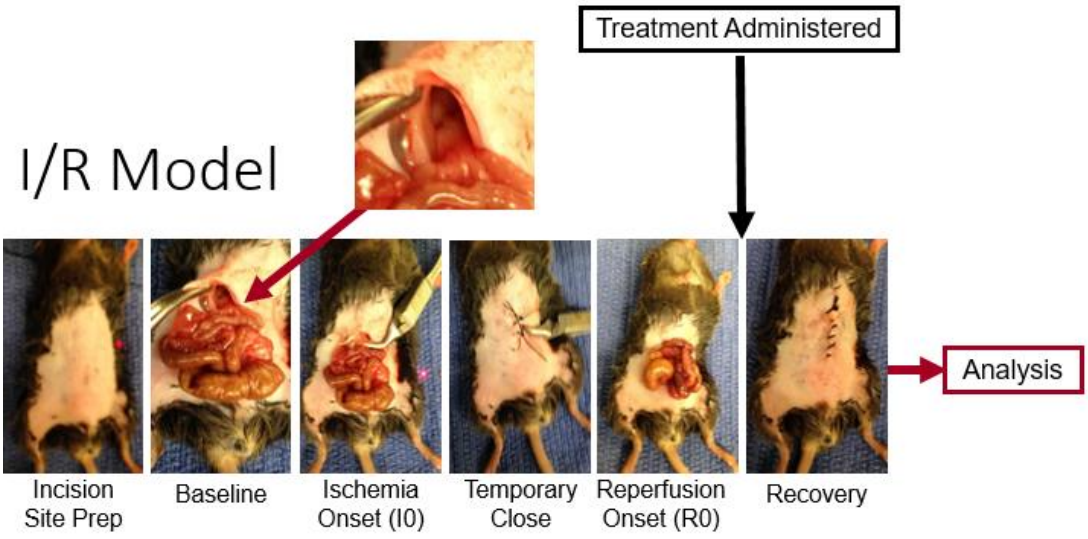

Figure 3. Murine Ischemia Reperfusion Injury Model

2.2 Survival Analysis

Animals assigned to the survival protocol were monitored twice daily for 7 days following surgery for death, pain, and incisional complications. End points of analysis included animal death or when Laboratory Animal Resource Center veterinarians felt animals were suffering and needed to be euthanized. Survival curves were created based on these end points. Remaining mice were euthanized with isoflurane overdose and cervical dislocation at completion of the 7 day designated time course.

2.3 Laser Doppler Imaging Analysis

Animals were assigned to the perfusion protocol (N=10 per vehicle group, 8 per each NaHS treated group) as follows: 1) WT IR+vehicle, 2) WT IR+2 nmol/kg NaHS, 3) WT IR+2 µmol/kg NaHS, 4) eNOS KO IR+vehicle, 5) eNOS KO IR+2 nmol/kg NaHS, and 6) eNOS KO IR+2 µmol/kg NaHS. Perfusion was analyzed using a Laser Doppler perfusion Imager (LDI; Moor Instruments, Wilmington, DE; Figure 4). Images were acquired at baseline, at the initial clamping of the superior mesenteric artery, and 24 hours after recovery. A region of interest was created around the entirety of exposed intestines to obtain a flux mean perfusion within this region. Three images were acquired at each time point and averaged. Perfusion data were expressed as a percentage of baseline (mean±SEM). After the 24-hour recovery analysis, animals were euthanized with isoflurane overdose and cervical dislocation, and intestinal tissues were explanted for further analyses.

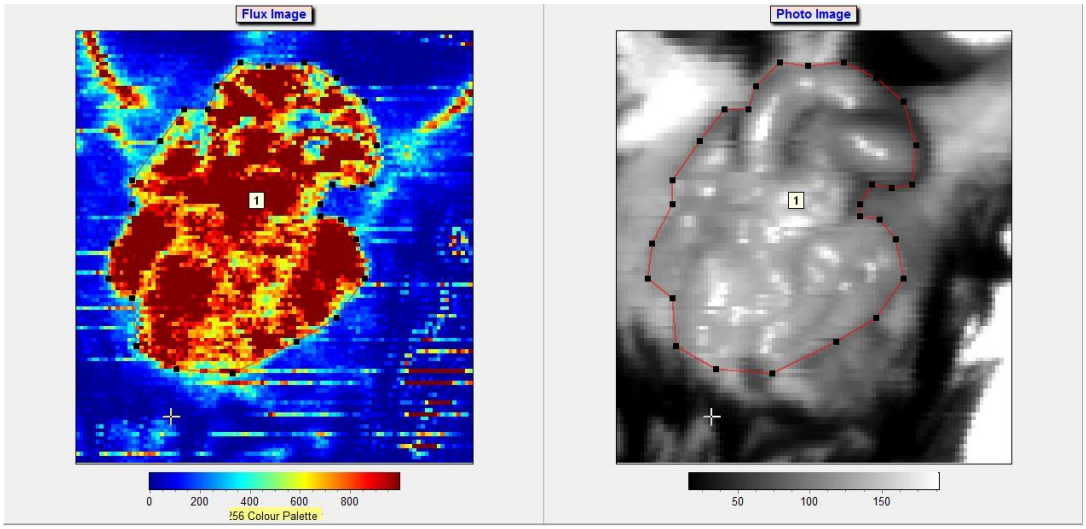

Figure 4. Intestinal Perfusion by Laser Doppler Imager.

2.4 Histological Analysis

Intestinal tissues were harvested following euthanasia of experimental groups. Terminal ileums were then fixed using 4% paraformaldehyde with subsequent

dehydration in 70% ethanol. Intestines were paraffin-embedded, sectioned, and subsequently stained with hematoxylin and eosin. A histological scoring method of intestinal damage was used as previously described: 0, no damage; 1, subepithelial space at the villous tip; 2, loss of mucosal lining at the villous tip; 3, loss of less than half of the villous structure; 4, loss of more than half of the villous structure; and 5, transmural necrosis [24] [25]. All histological sections were evaluated by two blinded authors (ARJ, NAD) and scores were averaged.

2.5 Inflammatory Cytokines

Following euthanasia, mouse intestinal tissues designated for protein analysis were harvested, snap frozen in liquid nitrogen and stored at -80ºC. Once ready to use, intestines were thawed and homogenized in RIPA buffer (Sigma, St. Louis, MO) with phosphatase and protease inhibitors (1:100 dilution, Sigma, St. Louis, MO) using a Bullet Blender tissue homogenizer (Next Advance, Averill Park, NY). Following homogenization, samples were centrifuged at 12,000 rpm to pellet extraneous tissue and supernatants were collected and placed into fresh Eppendorf tubes. Total protein concentration was quantified with the Bradford assay using a spectrophotometer (VersaMax microplate reader; Molecular Devices, Sunnyvale, CA).

Murine intestinal levels of interleukin 6 (IL-6), interleukin 9 (IL-9), interleukin 10 (IL-10), C-X-C ligand 10 (IP10), macrophage inflammatory protein 2 (MIP-2), granulocyte-colony stimulating factor (G-CSF), macrophage inflammatory protein-1 alpha (MIP-1α), eotaxin, vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), hepatocyte growth factor (HGF), and C-X-C ligand 1 (KC) were quantified using a Bio-Plex 200 multiplex beaded assay system (Bio-Rad, Hercules, Ca) with customizable multiplex plates for murine inflammatory cytokines (Millipore, Billerica, MA). Assays were performed at 1:20 dilution according to the manufacturer's

instructions and are reported in nanograms of cytokine per gram of total intestinal protein (mean±SEM).

2.6 Statistical Analysis

All statistical analysis was performed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA). Statistical significance for the survival studies was assessed by the Mantel-Cox log rank test and the Gehan-Breslow-Wilcoxon test. Perfusion, histology and cytokine analysis were reported as the mean±SEM and compared using the Mann-Whitney U test for nonparametric variables. P-values less than 0.05 were considered statistically significant.

CHAPTER THREE: RESULTS

3.1 H_2S Improves Survival Following Intestinal I/R Injury

NaHS promoted significant differences in survival based on the dose utilized. Survival was significantly improved in mice treated with low dose NaHS (2 nmol/kg, 83.3%) compared to mice that received vehicle control (40%, p=0.03, Figure 5). No difference was found between the 2 nmol/kg and 2 µmol/kg NaHS treated groups (p=0.68). Mice treated with 2 mmol/kg NaHS died immediately following intraperitoneal injection compared to vehicle control and did not awake from anesthesia (0%, p<0.005). Therefore, this higher dose was not used for further experiments.

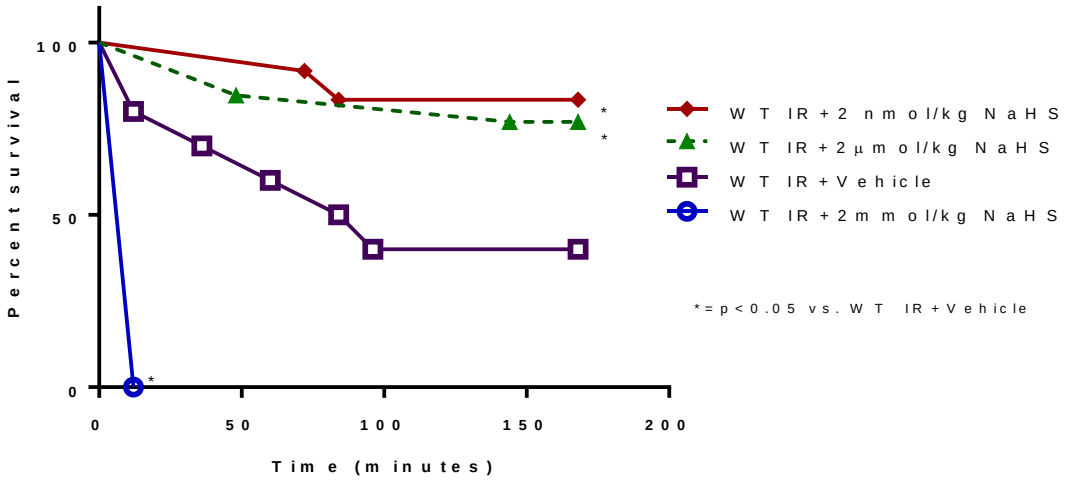

Figure 5. Dose Response Kaplan-Meier plots for 7 day survival after I/R injury. There was significantly improved survival following treatment with low dose NaHS (2 nmol/kg, p=0.03), and significantly worse survival compared to vehicle with the 2mmol/kg NaHS therapy (2mmol/kg, p=0.005) following intestinal I/R. Although use of the 2µmol/kg NaHS dose had no statistically significant difference from vehicle, it also had no statistically significant difference from the 2 nmol/kg group (*=p<0.05 versus vehicle).

3.2 H_2S Improves Intestinal Perfusion Following I/R Injury Through eNOS Dependent Pathways

Intestinal perfusion was compared in treatment groups at 24 hours following IR injury (Figure 6). Low-dose and high-dose NaHS treatment promoted significantly improved post-ischemic perfusion levels (78.3±7.3%, 63.4±7.2%) compared to vehicle (26.4±5.3%; p=0.0002, p=0.003 respectively) in WT animals assessed at 24 hours following injury. Conversely, in eNOS KO mice, perfusion was similar among treated and untreated groups, and no significant differences were found between vehicle (34.4±6.6%), 2 nmol/kg NaHS (32.1±6.9%) or 2μmol/kg NaHS (52.7±9.2%) treated groups (p=0.72 and p=0.33 respectively).

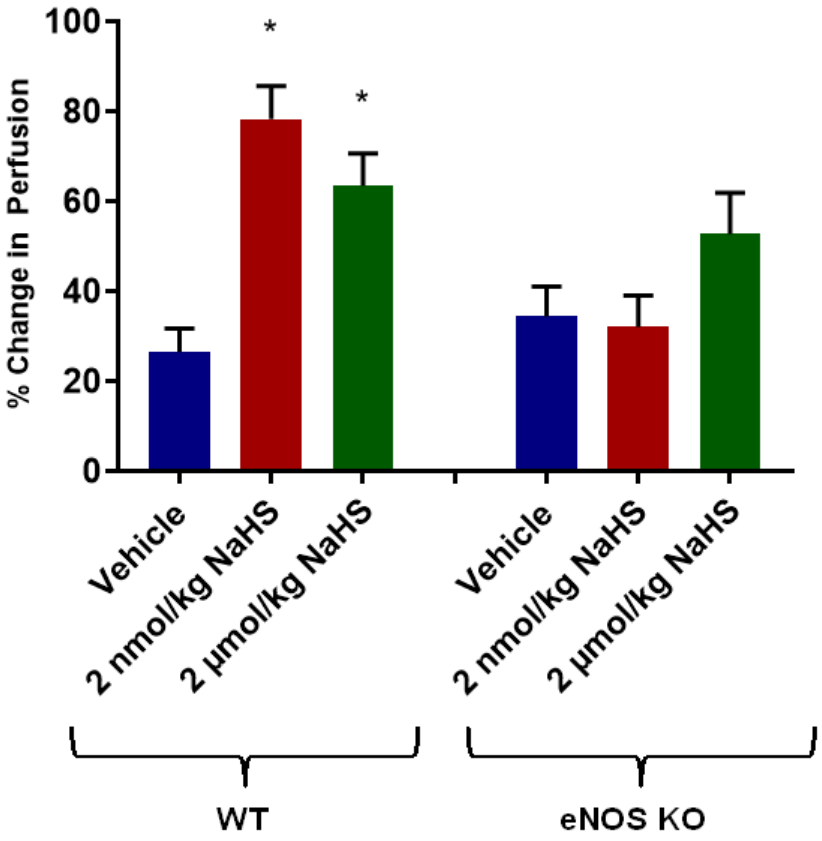

Figure 6: H_2S improves mesenteric perfusion following ischemic injury through eNOS dependent pathways. Post-ischemic mesenteric perfusion was significantly increased with both low dose and mid-range doses of NaHS in WT animals. However, there were no observed improvements in post-ischemic mesenteric perfusion following treatment in eNOS KO animals. (*=p<0.05 versus respective vehicle)

3.3 H_2S Prevents Mucosal Injury Following I/R Through eNOS Dependent Pathways

Significant sloughing of intestinal mucosa and destruction of the epithelial layer in the crypt-villous architecture was seen in PBS vehicle-treated WT groups (3.2±0.30) compared to WT mice treated with 2nmol/kg NaHS (1.3±0.25, p<0.0001) or 2µmol/kg NaHS (1.6±0.29, p=0.002). No significant improvements were seen in histological injury with NaHS therapy in eNOS KO animals (Figure 7).

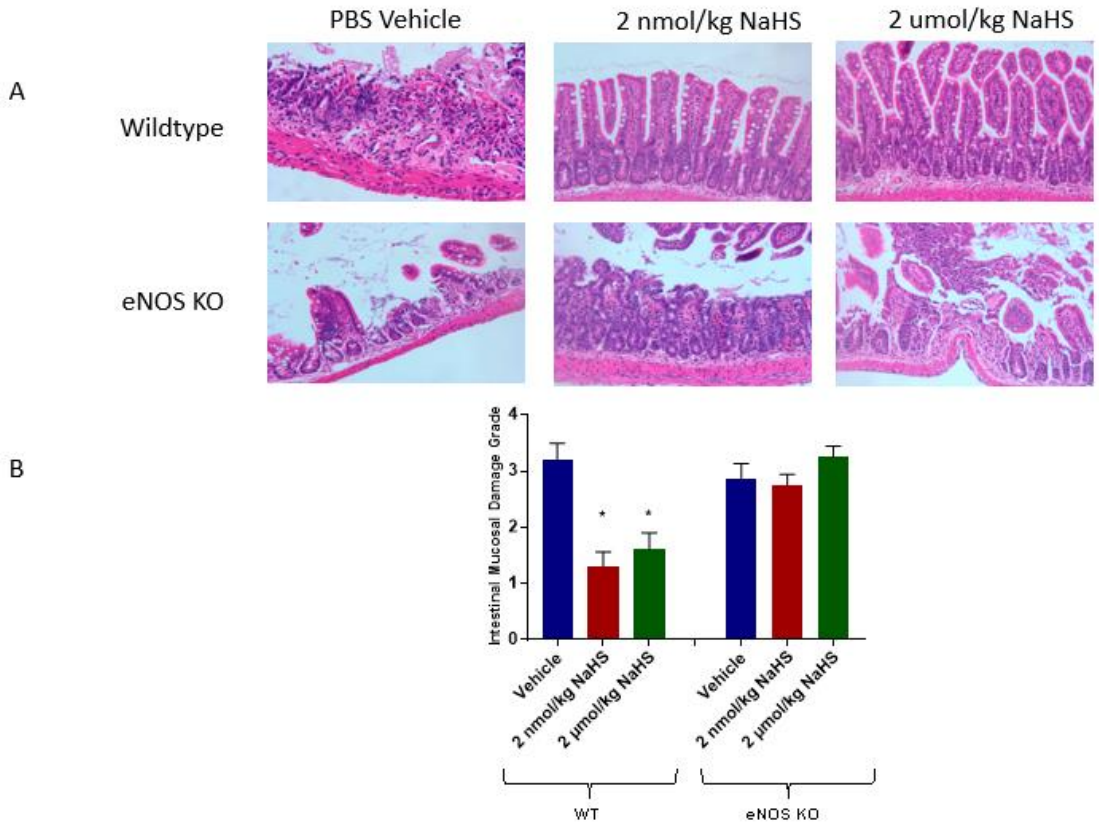

Figure 7: Histological examination of small intestine following intestinal I/R. A) Representative histology slides of each treatment group (hematoxylin and eosin stain, x20) demonstrating improvements in intestinal histology with NaHS therapy in WT animals but not in eNOS KO animals. B) Histological scoring of intestinal specimens (*= p<0.05 vs respective vehicle).

3.4 H_2S Attenuates the Intestinal Inflammatory Cytokine Response following I/R injury

Intestinal cytokines and growth factors were affected by hydrogen sulfide therapy following ischemic injury. IL-6 was noted to be significantly decreased in WT intestines exposed to both 2nmol/kg and 2µmol/kg NaHS, while IL-9, IL-10, VEGF, and FGF-2 were only lowered by the 2µmol/kg dose of NaHS (Figure 8). In murine animals with eNOS KO, while decreases in IL-9, IL-10, and VEGF were observed, they did not reach statistical significance. Interleukin 6 and FGF-2 were the only two cytokines to have a significant decreases in chemokine production. IL-6 significantly decreased with 2 µmol/kg NaHS treatment and FGF-2 significantly decreased with 2 nmol/kg NaHS treatment. HGF was not affected in this model system by NaHS or with eNOS ablation.

Inflammatory chemokines in the intestinal segments were also affected by NaHS following injury (Figure 9). MIP-1α, eotaxin, and IP-10 were significantly decreased only in the 2µmol/kg treated groups, while there was no effect on these factors with the lower doses of NaHS in WT intestinal segments. Levels of MIP-2, GCSF, and KC were significantly decreased in intestines with both tested doses of NaHS. Lastly, when evaluating cytokines from NaHS-treated intestines from eNOS KO animals, there were no significant differences compared to the vehicle-treated group.

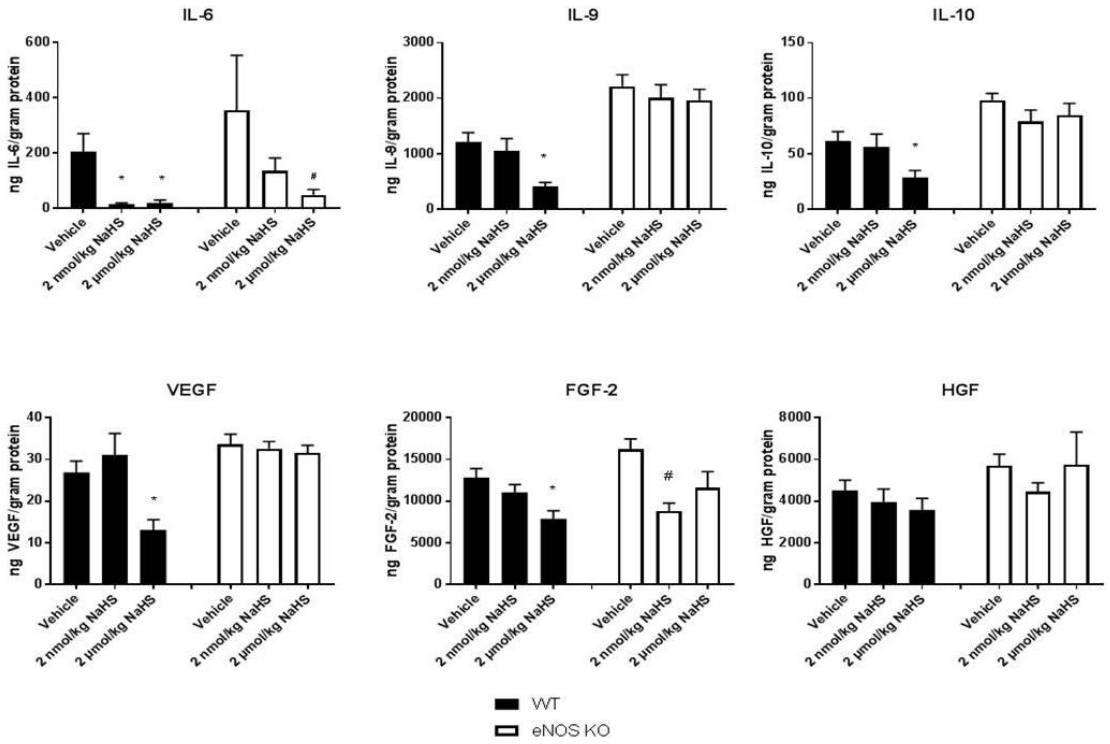

Figure 8: Inflammatory cytokines and growth factors are impacted by Hydrogen Sulfide therapy following Intestinal I/R. Intestinal levels of IL-6, IL-9, IL-10, VEGF, and FGF-2 are decreased with hydrogen sulfide therapy following intestinal ischemia. Hydrogen sulfide decreases in IL-9, IL-10, and VEGF appear to be facilitated by intact eNOS, whereas decreases in IL-6 and FGF-2 may work through H_2S independent pathways. HGF does not appear to be affected by hydrogen sulfide therapy or ablation of eNOS. (*=$p<0.05$ vs. WT vehicle, #=$p<0.05$ vs. eNOS KO vehicle)

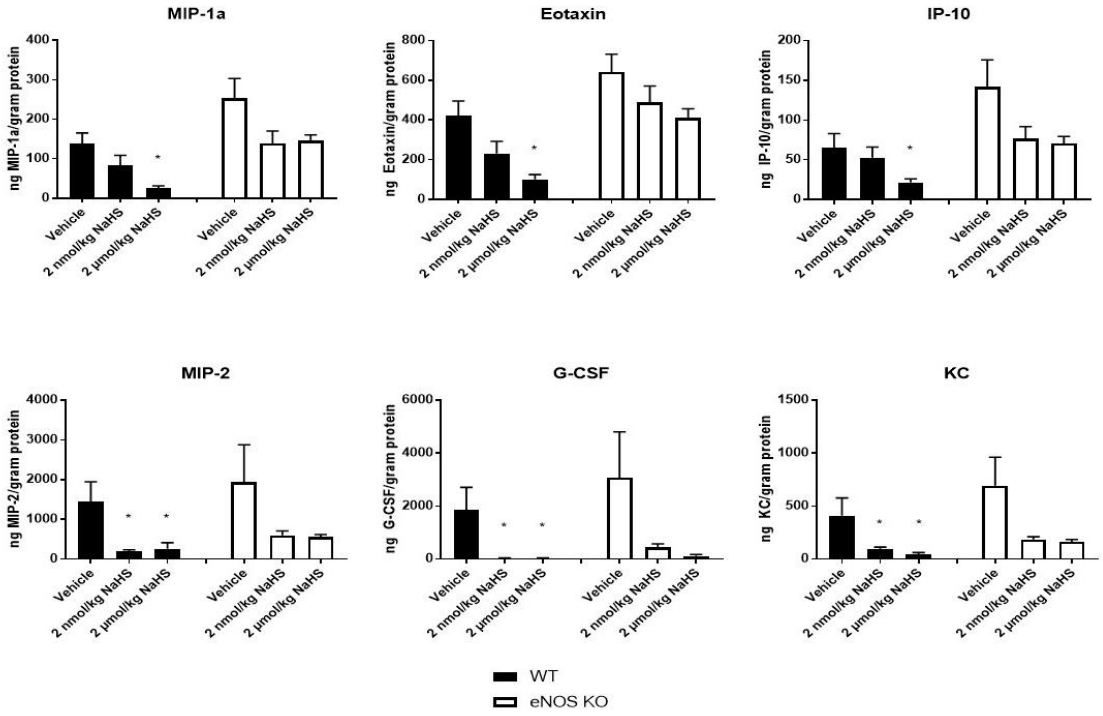

Figure 9: Chemokines are impacted by Hydrogen Sulfide therapy following Intestinal I/R. Intestinal levels of MIP-1α, eotaxin, IP-10, MIP 2, G-CSF, and KC are decreased in ischemic intestines following hydrogen sulfide therapy. Levels of these factors were not impacted significantly by genetic ablation of eNOS. (*=$p<0.05$ vs. WT vehicle)

CHAPTER FOUR: DISCUSSION & CONCLUSION

4.1 Discussion

Intestinal ischemia is a devastating abdominal emergency. Many times necrotic intestine needs to be surgically excised, which can leave patients with an inadequate amount of bowel to promote normal nutrient absorption. Current therapies are inadequate at protecting ischemic intestine, and therefore, novel therapies are being explored. Herein we observed that hydrogen sulfide treatment (in the form of NaHS) following ischemia and reperfusion injury can provide gastrointestinal protection. These beneficial properties appear to be mediated, in part, by endothelial nitric oxide synthase.

We observed improvements in survival, mesenteric perfusion, preservation of intestinal mucosal integrity and decreased inflammation following ischemic insult. However, while we did observe significant benefits with NaHS therapy following intestinal ischemia, these beneficial properties were not observed in the absence of endothelial nitric oxide synthase. Similarly, Coletta et al. previously reported that H_2S-induced angiogensis requires nitric oxide biosynthesis and inhibition of eNOS abolished the H_2S-stimulated angiogenic response [26]. Their research suggested that nitric oxide and H_2S worked synergistically to increase cGMP production, cGMP-dependent protein kinase G (PKG) activation, and angiogenesis. Additionally, in their studies the vasorelaxant action of H_2S also required the presence of endogenously produced nitric oxide, further confirming the significant complexity of biological interactions between these two gasotransmitters.

NaHS clearly impacted survival, although this appeared to be in a reverse dose dependent fashion, with higher doses promoting near instantaneous death. This certainly makes sense given that hydrogen sulfide is toxic in high doses. Other studies have corroborated toxic effects on organs at similar millimolar doses used in this study [27]. Toxicity is felt to result from inhibition of cytochrome C oxidase in complex IV of the

electron transport chain, the last complex prior to ATP production [28]. The resulting effect is cardiovascular demise with a rapid fall in left ventricular ejection fraction and pulseless electrical activity [29].

However, at more physiologic doses, sulfide ions are donated to complex II of the mitochondrial electron transport chain which assists in stimulating ATP production [28]. In addition, it appears that hydrogen sulfide S-sulfhydrates eNOS at Cys^{443} which promotes dimerization of eNOS and production of nitric oxide [18]. Nitric oxide then promotes vasodilation. In this study, we observed equivalent rates of improvement in mesenteric perfusion with the addition of both 2nmol/kg and 2μM/kg NaHS. These improvements in perfusion were lost when eNOS was genetically ablated.

Intestinal mucosal injury was also improved with NaHS therapy. Although it is possible that H_2S can directly preserve intestinal mucosal integrity by limiting inflammation and apoptosis [11, 12, 17], it is also equally likely that the main effects of hydrogen sulfide therapy are to promote vasodilation [30] and improve mesenteric blood flow. Better intestinal reperfusion after injury in itself will limit further mucosal damage and intestinal inflammation. Furthermore, if the effects of H_2S were solely on the intestinal epithelium, then knocking out endothelial nitric oxide synthase should not have had an effect on host preservation.

Significant elevations in intestinal chemokines, including macrophage inflammatory protein (MIP), eotaxin, and chemokine ligand 10 (IP-10) have been noted following intestinal ischemia, and are thought to be responsible for leukocyte mobilization to the areas of injured bowel [31, 32]. These cells are responsible for injury repair, but also promote inflammation, which may be detrimental to the host. For example, lymphocyte influx is thought to be detrimental to recovery, as lymphocyte depleted animals had better outcomes following intestinal ischemia [33]. However, other

leukocyte classes may actually promote intestinal recovery by digesting dead cells and repairing the extracellular matrix [34]. Additionally, markers of neovascularization have been elevated after intestinal ischemia [35, 36]. Neovasculogenesis increases intestinal capillary density to restore oxygen balance and nutrient homeostasis to injured bowel.

In similar fashion, we also appreciated a significant decrease in intestinal chemokines. High dose NaHS (2 μmol/kg) decreased MIP-1α, eotaxin, and IP-10 levels, while both low and high-range doses significantly decreased intestinal MIP-2, GCSF, and KC levels. While we observed lower levels of several chemokines in eNOS KO animals following NaHS therapy, none of these reached statistical significance. Therefore, hydrogen sulfide mediated decreases in all assessed chemokines following intestinal ischemia appeared to be dependent on the presence of eNOS.

We also observed a significant decrease in several inflammatory cytokines and growth factors after NaHS therapy. IL-6, IL-9, IL-10, VEGF, and FGF-2 were all decreased after NaHS therapy, although only IL-6 was noted to be decreased at both the 2 nmol/kg and 2 μmol/kg concentrations. HGF was not impacted by NaHS therapy. While decreases in IL-9, IL-10, and VEGF appeared to be dependent on functioning eNOS, production of IL-6 appeared to also decrease in mid-range dose eNOS KO animals, and FGF-2 appeared to decrease in the low-range NaHS treated eNOS KO animals. HGF was also not affected by ablation of eNOS. These findings would indicate that many of the inflammatory factors tested, but certainly not all of them, were impacted by the presence of functional eNOS. Based on the data presented herein, we surmise that the presence of eNOS promotes hydrogen sulfide mediated improvements in vascular tonicity. Because of better mesenteric perfusion, injured intestines are able to recover more effectively and inflammatory cytokines and chemokines are not as abundant.

4.2 Limitations

The superior mesenteric artery (SMA) ligation model of intestinal I/R does not model clinical intestinal ischemia to its fullest. Although complete small bowel ischemia is possible secondary to SMA thrombus or embolus, the majority of intestinal ischemic episodes are due to segmental intestinal ischemia, such as may be seen with adhesive bowel obstructions or incarcerated hernias. Nonetheless, this model mimics the most severe form of intestinal ischemia, and therefore, is likely considered the best animal model available to test the effectiveness of novel therapies.

4.3 Conclusions

In conclusion, hydrogen sulfide appears to be a novel therapeutic agent for the treatment of intestinal ischemia and reperfusion injury. The beneficial effects of improved mesenteric perfusion, histological injury, and inflammatory cytokine and chemokine modulation appear to be mediated through endothelial nitric oxide synthase. Additional modes of delivery, including inhaled delivery systems, need to be tested with this model to determine best clinical application. These factors, as well as the effects of hydrogen sulfide on long term survival and neurodevelopment, need to be assessed prior to widespread clinical implementation.

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Curriculum Vitae

Amanda R Jensen, MD

EDUCATION

August 2015 -Present	Master's in Translational Science Indiana University–Purdue University Indianapolis Anticipated completion: 2018
August 2009 - May 2013	Doctor of Medicine Sanford School of Medicine University of South Dakota Vermillion, SD
August 2005 - May 2008	Buena Vista University Storm Lake, IA B.S., Biology with Minor in Chemistry

POST-GRADUATE TRAINING

July 2015-June 2017	Surgical Research Fellow Indiana University Department of Surgery *Interest: Intestinal Ischemia-Reperfusion Injury*
July 2013-Present	General Surgery Residency Indiana University Department of Surgery Indianapolis, IN *Anticipated completion: 2020*

MEDICAL LICENSURE, CERTIFICATION & SPECIALTY BOARD STATUS

2013-Present	Indiana Medical License - 01077022A
July 2013	Advanced Trauma Life Support (ATLS)
July 2013, February 2017	Advanced Cardiac Life Support (ACLS)
July 2013	Pediatric Advanced Life Support (PALS)
July 2013, July 2016	Basic Life Support (BLS)

PROFESSIONAL HONORS AND AWARDS

April 2016	The Carl H. McCaskey Award (Subspecialty Surgery) America College of Surgeons – Indiana Chapter Annual Meeting
February 2016	Alpha Omega Alpha Honor Medical Society
August 2008	Undergraduate Rural Medicine Education and Development (URMED) Internship at the Buena Vista Regional Medical Center

September 2007 — 3rd Place Iowa and Nebraska Physiological Societies Annual Meeting

May-August 2007 — Buena Vista Undergraduate Research Fellowship

PROFESSIONAL DEVELOPMENT

2017 — American College of Surgeons Clinical Congress, San Diego, CA

2017 — American Academy of Pediatrics National Conference and Exhibition, Chicago, IL

2017 — Pacific Association of Pediatric Surgeons, Seattle, WA

2017 — Annual American Pediatric Surgical Association Conference, Hollywood, FL

2017 — NEC Symposium, Sacramento, CA

2017 — 11th Annual Academic Surgical Congress, Las Vegas, NV

2016 — Annual American Pediatric Surgical Association Conference, San Diego, CA

2016 — 39th Annual SHOCK Society National Meeting, Austin, TX

2016 — 7th Annual International Women in Surgery Career Symposium, Orlando, FL

2015 — American Academy of Pediatrics National Conference, Washington D.C.

2012 — Annual American Pediatric Surgical Association Conference, San Antonio, TX

2011 — Pediatric Academic Societies and Asian Society for Pediatric Research Joint Meeting, Denver, CO

RESEARCH EXPERIENCE

2015 – 2017 — Translational Science Research – Surgical Research Fellow
Mentor: Dr. Troy Markel, MD, Pediatric Surgeon, Riley Hospital for Children at Indiana University Health

- Investigation of ischemia-reperfusion injury and therapeutic treatment with human mesenchymal stromal cells; role of hydrogen sulfide

2011	Medical Student Researcher Scholarship Pathways Program Mentor: Dr. Casas-Melley, Pediatric Surgeon, Sanford Children's Hospital • Retrospective review of Nissen fundoplication outcomes in children
2010	Medical Student Researcher SPR/APS Student Research Program Mentor: Dr. Catherine Gordon, MD MSc, Children's Hospital Boston • Retrospective review of Depot Medroxyprogesterone Acetate use and Bone Mineral Density loss
2006-2008	Student Researcher Buena Vista University, Storm Lake, IA Mentor: Dr. Robert Dunbar • Explored chloride dynamics in mouse hippocampal neurons through the use of confocal microscopy and transgenic mice expressing a chloride sensitive, dimeric protein Clomeleon

PROFESSIONAL ORGANIZATION MEMBERSHIPS

2016-Present	Association for Academic Surgery *Resident Member*
2016-Present	Association of Women in Surgery *Resident Member*
2016-Present	American Pediatric Surgical Association *Resident Member*
2013-Present	American College of Surgeons (Indiana Chapter) *Resident Member*

SERVICE

UNIVERISTY SERVICE

July 2016- July 2017	Indiana University School of Medicine House Staff Forum – Chair
July 2015 – July 2017	Indiana University Annual Program Evaluation/A3 Committee
July 2015 – July 2017	Indiana University General Medical Education Committee (GMEC)
July 2015 – July 2017	Indiana University School of Medicine LCME Accreditation Committee

July 2015 – July 2017 Indiana University School of Medicine House Staff Forum, Member

PROGRAM SERVICE

July 2015 – Present Indiana University General Surgery Program Evaluation Committee

July 2014 – Present Indiana University General Surgery Program Resident Council

JOURNAL SERVICE

January 2017 – Present Reviewer, Journal of Surgical Research

ABSTRACTS & PRESENTATIONS

POSTER PRESENTATIONS

National/International Meetings

May 2017 **Jensen AR**, Drucker NA, Khaneki S, Markel TA. Direct Peritoneal Application of Hydrogen Sulfide is Superior to Inhaled Hydrogen Sulfide Gas Following Intestinal Ischemia and Reperfusion Injury

Pacific Association of Pediatric Surgeons

May 2017 **Jensen AR**, Renaud ER, Drucker NA, Staszak J, Senay A, Umesh V, Williams RF, Markel TA. Why Wait? : Early Enteral Feeding After Pediatric Gastrostomy Tube Placement

American Pediatric Surgical Association 2017 Annual Meeting

May 2017 **Jensen AR**, Drucker NA, Khaneki S, and Markel TA. Umbilical Stromal Cells Mediate Intestinal Protection Following Ischemia/Reperfusion Injury by Nitric Oxide Dependent Pathways

American Pediatric Surgical Association 2017 Annual Meeting

April 2017	Drucker NA, **Jensen AR**, Khaneki S, Ferkowicz MJ and Markel TA. Extended Release Hydrogen Sulfide Donor is Protective in Murine Model of Necrotizing Enterocolitis *Necrotizing Enterocolitis Symposium*
June 2016	**Jensen AR**, Manning MM, Khaneki S, Markel TA. Hydrogen Sulfide Improves Survival, Mesenteric Perfusion, and Intestinal Integrity Following Ischemia/Reperfusion Injury *39th Annual Shock Society Meeting*
April 2011	**Dorale AR**, Pitts SA, Feldman HA and Gordon CM. Depo Medroxyprogesterone Acetate (DMPA) and Bone Density in Adolescents and Young Adults: A Retrospective Review. *Pediatric Academic Societies and Asian Society for Pediatric Research Joint Meeting*
Local/Regional Meetings	
May 2013	**Dorale AR**, Ryckman J, Pearce D, Thompson P, Casas-Melley A. Longitudinal Outcomes of Laparoscopic Nissen Fundoplication in Adolescents, Children and Infants. Sanford School of Medicine. University of South Dakota *Scholarship Pathways Program*
February 2012	**Dorale AR.** WIC: Improving Nutrition with Healthy Choices. Sanford School of Medicine. University of South Dakota *Beyond Borders: A Cultural Immersion Experience Cultural Colloquium*
2007-2008	**Dorale AR**, Glienke KJ, and Dunbar RL. Imaging of Intracellular Chloride Changes in Mouse Hippocampal Neurons During Bath Application of a GABAA Agonist. Department of Biology, Buena Vista University. Storm Lake, IA. *Iowa Academy of Science* *Buena Vista Scholar's Day* *The Combined Iowa and Nebraska Physiological Societies Annual Meeting*

ORAL PRESENTATIONS

National/International Meetings

October 2017	Drucker NA, **Jensen AR**, Khaneki S, Ferkowicz MJ and Markel TA. Hydrogen Sulifide Protects the Intestine in a Murine Model of Necrotizing Enterocolitis. *American College of Surgeons Clinical Congress*
September 2017	**Jensen AR**, Drucker NA, Ferkowicz MJ and Markel TA. Post-ischemic Intraperitoneal Hydrogen Sulfide Therapy Is Superior to Intravenous or Inhaled Hydrogen Sulfide Gas Following Intestinal Ischemia and Reperfusion Injury. *American Academy of Pediatrics National Conference & Exhibition*
September 2017	**Jensen AR**, Drucker NA, Ferkowicz MJ and Markel TA. Loss of Endothelial Nitric Oxide Synthase Exacerbates Intestinal Injury and Systemic Inflammation in Experimental Necrotizing Enterocolitis. *American Academy of Pediatrics National Conference & Exhibition*
February 2017	Khaneki S, **Jensen AR**, Drucker NA, Markel TA. Direct Peritoneal Resuscitation Improves Mesenteric Perfusion by Nitric Oxide Dependent Pathways *12th Annual Academic Surgical Congress*
February 2017	**Jensen A**, Drucker NA, Khaneki S, Markel TA. H_2S Improves Intestinal Perfusion and Integrity After Ischemia by Nitric Oxide Dependent Pathways *12th Annual Academic Surgical Congress* *AAS Resident/Fellow Competition*
August 2016	Choi JN, Nickel BL, Canal D, **Jensen AR**, Torbeck L. Impact of Integrated Residencies on General Surgery Operative Volume. *Midwest Surgical Association Annual Meeting*

May 2016	**Jensen AR**, Manning MM, Khaneki S, Markel TA. Stromal Cell Source Does Not Impact Survival or Mesenteric Perfusion Following Intestinal Ischemia *American Pediatric Surgical Association Annual Meeting*
October 2015	**Jensen AR**, Markel TA. Circulating Mesenchymal and Hematopoietic Stem Cells Are Elevated in Children Following Traumatic Injury *2015 American Academy of Pediatrics (AAP) National Conference & Exhibition*
April 2011	**Dorale AR**, Pitts SA, Feldman HA and Gordon CM. Depo Medroxyprogesterone Acetate (DMPA) and Bone Density in Adolescents and Young Adults: A Retrospective Review. *Pediatric Academic Societies and Asian Society for Pediatric Research Joint Meeting*

Local/Regional Meetings

October 2017	**Jensen AR.** Hyperparathyroidism. *Resident Education Hour at Indiana University School of Medicine*
May 2017	**Jensen AR.** Mesenchymal stromal cells provide intestinal recovery following ischemia reperfusion injury through hydrogen sulfide production and secondary nitric oxide dependent pathways. *37th Annual Resident Research Day IU Department of Surgery*
April 2017	**Jensen AR**, Drucker NA, Khaneki S, Markel TA. Umbilical Stromal Cells Improve Intestinal Recovery Following Ischemia/Reperfusion Injury by Nitric Oxide Dependent Pathways *Indiana Chapter – American College of Surgeons Annual Meeting*

April 2016	**Jensen AR**, Markel TA. Stromal Cell Source Does Not Impact Survival or Mesenteric Perfusion Following Intestinal Ischemia Reperfusion Injury. *Indiana Chapter – American College of Surgeons Annual Meeting*
February 2015	Rectal Cancer: Down to Up – Up to Down *John E. Jesseph Visiting Professor Resident Education Hour with Dr. Peter Marcello, MD, FACS, FASCRS at Indiana University School of Medicine*
January 2015	Sepsis/SIRS Syndrome *Resident Education Hour at Indiana University School of Medicine*

PUBLISHED MANUSCRIPTS

1. **Jensen AR**, Renaud ER, Drucker NA, Staszak J, Senay A, Umesh V, Williams RF, Markel TA. Why Wait? : Early Enteral Feeding After Pediatric Gastrostomy Tube Placement. J Pediatr Surg. 2017 June 27. PMID: 28689884
2. **Jensen AR**, Drucker NA, Khaneki S, Ferkowicz MJ, Yoder MC, DeLeon ER, Olson KR, and Markel TA. Hydrogen Sulfide: A Potential Novel Therapy for the Treatment of Ischemia. Shock. 2017 Nov; 48(5): 511-524. PMID: 28498298
3. **Jensen AR**, Drucker NA, Khaneki S, Ferkowicz MJ, Markel TA. Hydrogen Sulfide Improves Intestinal Recovery Following Ischemia by Endothelial Nitric Oxide Dependent Mechanisms. Am J Physiol Gastrointest Liver Physiol. 2017 Mar 9:ajpgi.00444.2016 [Epub ahead of print] PMID: 28280145
4. Khaneki S, **Jensen AR**, Drucker NA, Markel TA. Direct Peritoneal Resuscitation Improves Mesenteric Perfusion by Nitric Oxide Dependent Pathways. Journal of Surgical Research. 2017 June; 213: 274-280. PMID: 28601326
5. **Jensen AR**, Nickel BL, Dolejs SC, Canal DF, Torbeck L, Choi JN. Impact of Integrated Programs on General Surgery Operative Volume. Am J Surg. 2017 Feb; 213(2):346-352. PMID: 27955883
6. Doster DL, **Jensen AR**, Khaneki S, Markel TA. Mesenchymal Stromal Cell Therapy for the Treatment of Intestinal Ischemia: Defining the Optimal Cell Line for Maximum Therapeutic Benefit. Cytotherapy, 2016;18(12):1457-1470. PMID: 27745788
7. **Jensen AR**, Manning MM, Khaneki S, Drucker NA, Markel TA. Harvest Tissue Source Does Not Alter the Protective Power of Stromal Cell Therapy Following Intestinal Ischemia and Reperfusion Injury. Journal of Surgical Research. 2016 Aug; 204(2):361-70. PMCID: PMC5002874

8. **Jensen AR**, Baertschiger RM, Hackworth J, Rescorla FJ. Blunt Abdominal Trauma with Handlebar Injury: A Rare Cause of Traumatic Amputation of the Appendix Associated with Acute Appendicitis. Journal of Pediatric Surgery Case Reports. 2016 April; 7:13-15.
9. **Jensen AR**, Doster DL, Hunsberger EB, Manning MM, Stokes SM, Barwinska D, March KL, Yoder MC, Markel TA. Human Adipose Stromal Cells Increase Survival and Mesenteric Perfusion Following Intestinal Ischemia and Reperfusion Injury. Shock. 2016 Jul; 46(1):75-82. PMID: 26796571
10. Markel TA, Crafts TD, **Jensen AR**, Hunsberger EB, Yoder MC. Human Mesenchymal Stromal Cells Decrease Mortality Following Intestinal Ischemia and Reperfusion Injury. Journal of Surgical Research. 2015 Nov; 199(1):56-66. PMID: 26219205
11. Crafts TD, Hunsberger EB, **Jensen AR**, Rescorla FJ, Yoder MC, Markel TA. Direct peritoneal resuscitation improves survival and decreases inflammation after intestinal ischemia and reperfusion injury. Journal of Surgical Research. 2015 Dec; 199(2):428-34. PMID: 26169030
12. Crafts TD, **Jensen AR**, Blocher-Smith EC, Markel T. Vascular Endothelial Growth Factor: Therapeutic Possibilities and Challenges for the Treatment of Ischemia. Cytokine. 2015 Feb; 71(2):385-393. PMID: 25240960
13. Savoie KB, Aziz SK, Blakely ML, Dassinger S, **Dorale AR**, Duggan EM, Harting MT, Huang EY, Markel TA, Moore-Olufemi SD, Shah SR, St. Peter SD, Tsao K, Wyrick DL, Williams RF. Improving gastroschisis outcomes: does birthplace matter? J Pediatr Surg. 2014 Dec;49(12):1771-5. PMID:25487481
14. Pitts SA, Feldman HA, **Dorale A**, and Gordon CM. Bone Mineral Density, Fracture, and Vitamin D in Adolescents and Young Women using Depot Medroxyprogesterone Acetate. Journal of Pediatric and Adolescent Gynecology. 2012 Feb; 25 (1):23-6. PMID: 22078997